A Functional Comparison of CD34$^+$CD38$^-$ Cells in Cord Blood and Bone Marrow

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We present cell cycling and functional evidence that the CD34$^+$CD38$^-$ immunophenotype can be used to define a rare and primitive subpopulation of progenitor cells in umbilical cord blood. CD34$^+$CD38$^-$ cells comprise 0.05% ± 0.08% of the mononuclear cells present in cord blood. Cell cycle analysis with the fluorescent DNA stain 7-aminoactinomycin D showed that the percentage of CD34$^+$ cells in cycle directly correlated with increasing CD38 expression. CD34$^+$CD38$^-$ cord blood cells were enriched for long-term culture-initiating cells (LTCIC; cells able to generate colony-forming unit-cells [CFU-C] after 35 to 60 days of coculture with bone marrow stroma) relative to CD34$^+$CD38$^+$ cells. In an extended LTCIC assay, CD34$^+$CD38$^-$ cells were able to generate CFU-C between days 60 and 100, clearly distinguishing them from CD34$^+$CD38$^+$ cells that did not generate CFU-C beyond day 40. When plated as single cells, onset of clonal proliferation was markedly delayed in a subpopulation of CD34$^+$CD38$^-$ cells; clones (defined as >100 cells) appeared after 60 days of culture in 2.9% of CD34$^+$CD38$^-$ cells. In contrast, 100% of CD34$^+$CD38$^+$ cells formed clones by day 21. Although the CD34$^+$CD38$^-$ immunophenotype defines highly primitive populations in both bone marrow and cord blood, important functional differences exist between the two sources. CD34$^+$CD38$^-$ cord blood cells have a higher cloning efficiency, proliferate more rapidly in response to cytokine stimulation, and generate approximately sevenfold more progeny than do their counterparts in bone marrow.

UMBILICAL CORD BLOOD is attracting increasing interest as a source of hematopoietic stem cells (HSC) for gene therapy. Although previous studies have provided functional characterization of the committed progenitor (CD34$^+$) population in cord blood, relatively little is known about the more primitive subpopulations present in cord blood that are enriched for HSC.[1-3] The immunophenotypic characterization and determination of the cycling and growth characteristics of the most primitive progenitors present in cord blood are essential steps toward assessment of cord blood HSC as potential targets for gene therapy.

One immunophenotype commonly used to identify HSC in adult bone marrow has been found not to apply to cord blood HSC. CD34$^+$ bone marrow cells with low or absent expression of HLA-Dr antigen are enriched for primitive blast-cell–containing colonies, high proliferative potential–colony-forming cells (HPP-CFC), and long-term culture-initiating cells (LTCIC).[4-6] However, in cord blood, the converse appears to apply, ie, the HLA-Dr$^+$ subpopulation of CD34$^+$ cells is more primitive than the CD34$^+$HLA-Dr$^-$ population.[7]

The CD34$^+$CD38$^-$ immunophenotype defines a primitive subpopulation of progenitor cells in fetal liver, fetal bone marrow, and adult bone marrow.[8-13] In this report, we have studied the cell cycle status and generative capacity of CD34$^+$CD38$^-$ and CD34$^-$CD38$^-$ cells in cord blood and compared them with their counterparts in adult bone marrow. The CD34$^+$CD38$^-$ immunophenotype defines a rare, quiescent subpopulation in both cord blood and bone marrow that can be distinguished functionally from the CD34$^+$CD38$^+$ population by sustained clonogenicity in an extended long-term culture assay. Late onset of proliferation from a small percentage of CD34$^+$CD38$^-$ cells contributes to the prolonged generation of colony-forming unit-cells (CFU-C) for 60 to 100 days of long-term culture. Although the CD34$^+$CD38$^-$ immunophenotype is consistent in both cord blood and bone marrow in defining the most primitive progenitors, functional differences between CD34$^+$CD38$^-$ cells from the two sources exist that have important implications for gene therapy.

MATERIALS AND METHODS

Cell sources. Bone marrow was obtained from consenting healthy adult volunteers by aspiration from the posterior iliac crest. Umbilical cord blood was obtained after vaginal and cesarean deliveries at Kaiser Permanente Hospital Sunset (Los Angeles, CA) after clamping and cutting of the cord by drainage of blood into sterile collection tubes containing the anticoagulant citrate-phosphate-dextrose (Sigma, St Louis, MO). All bone marrow and cord blood specimens were obtained according to guidelines approved by the Childrens Hospital Los Angeles Committee on Clinical Investigation. Cells were processed within 24 hours of collection.

Fluorescent antibody labeling and cell sorting. Mononuclear cells (MNC) were isolated from bone marrow and cord blood using Ficoll Hypaque (Pharmacia, Piscataway, NJ) density centrifugation. After lysis of residual red blood cells with Ortho Lysis buffer (Ortho Diagnostic Systems, Inc, Raritan, NJ), the mononuclear cells were washed and resuspended in cold (4°C) phosphate-buffered saline (PBS) at a concentration of 10^6 cells per 100 μL for incubation with fluorescent-labeled antibodies. In each of the cell sorting experiments, 2×10^6 cells (200 μL) were incubated for 30 minutes at 4°C in 20 μL undiluted fluorescein isothiocyanate (FITC)-labeled anti-CD34 (FITC-HPCA2; Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) and 20 μL undiluted phycoerythrin (PE)-conjugated anti-CD38 (PE-leu 17; BDIS). Aliquots of 10^6 cells used for isotype controls were incubated for 30 minutes in 50 μL of FITC-murine IgG (diluted 1:100; Coulter, Hialeah, FL) and 50 μL of PE-murine IgG (diluted 1:50). After incubation, cells were washed once in PBS. All fluorescence-activated cell sorting (FACS) analysis and cell sorting was performed on a FACSVantage (BDIS) equipped with an argon laser tuned to 488 nm. To determine the frequency of CD34$^+$ subpopulations in each sample, 50,000 events were acquired in listmode data file. Further analysis was performed on cells

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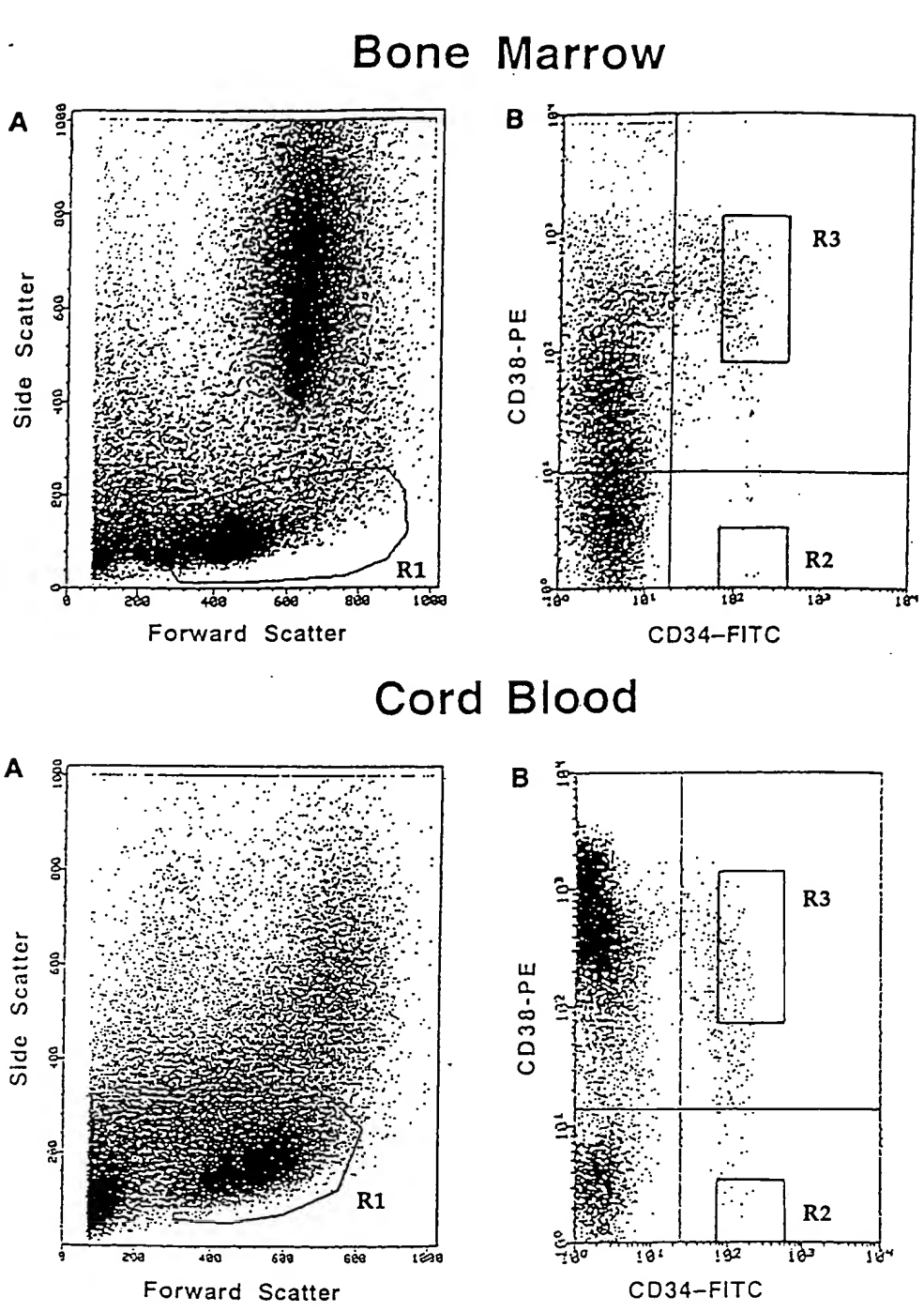

Fig 1. FACS analysis of cord blood and bone marrow MNC. (A) Forward scatter (size) and side scatter (density) of bone marrow and cord blood MNC is shown showing region R1 in which CD34⁺ progenitor cells are located. (B) CD34 and CD38 expression of bone marrow and cord blood cells from region R1. Quadrants are defined by FITC- and PE-labeled isotype controls (see Materials and Methods). Region R2, used to define CD34⁺CD38⁻ cells for sorting experiments, is defined as CD34⁺ cells with PE-CD38 fluorescence less than half maximum PE fluorescence of the isotype control. Region R3 was used to define CD34⁺CD38⁺ cells for sorting experiments.

after gating on the lymphoid region (R1 in Fig 1A). Quadrants were defined using isotype controls. In the cell sorting experiments, $CD34^+CD38^-$ cells were defined strictly as those with high CD34 antigen expression and anti-CD38 PE fluorescence less than half of the maximum PE fluorescence of the isotype control (R2 in Fig 1B).

To analyze the HLA-Dr expression of the $CD34^+CD38^-$ population, cells were incubated with FITC-CD34, PE-CD38, and anti-HLA-Dr directly labeled with peridinin chlorophyll protein (PerCP; BDIS) and analyzed by FACS using an argon laser.

FACS analysis of cell cycling. 7-amino actinomycin D (7-AAD) is a fluorescent DNA stain with an emission spectrum that allows its use in triple color analysis in combination with FITC- and PE-conjugated monoclonal antibodies.[14-16] To analyze cell cycle status of $CD34^+$ subsets, cells were initially incubated with FITC–anti-CD34 and PE–anti-CD38 or with the isotype controls as described above and then fixed in cold 0.5% paraformaldehyde (Sigma) for 60 minutes at 4°C. They were then washed once in PBS, resuspended in 0.05% Tween 20 (Aldrich Chemical, Milwaukee, WI) at a cell concentration of 5×10^6/mL, and incubated at 37°C for 15 minutes. After permeablization, cells were washed twice in PBS and resuspended in 500 μL of PBS with 25 μL of 500 μg/mL 7-AAD (Molecular Probes Inc, Eugene, OR) per 10^6 cells. The cells were incubated in 7-AAD for 20 minutes at room temperature and then immediately analyzed by FACS. Cell cycle analysis was performed using a FACSVantage equipped with an argon laser tuned at 488 nm and PC Lysys software program (BDIS). Fifty thousand to 200,000 events

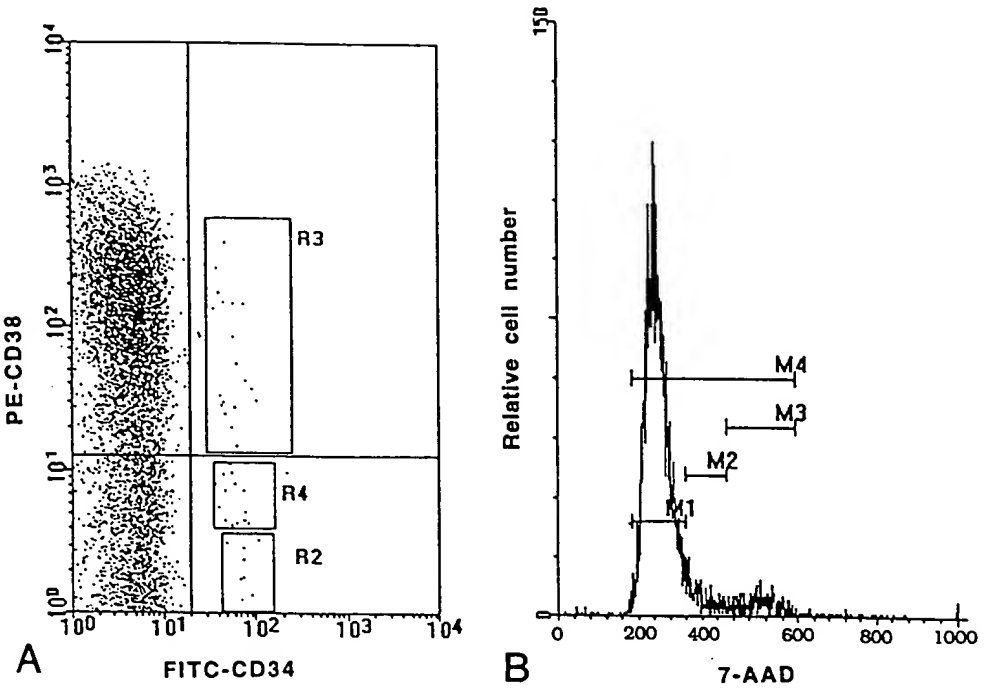

Fig 2. 7-AAD cell cycling assay. (A) FACS analysis showing CD34 and CD38 expression of MNC (from region R1, Fig 1A) that have been stained with the DNA fluorescent stain 7-AAD. Quadrant markers are defined by FITC and PE fluorescence of isotype controls that have also been stained with the DNA stain 7-AAD. Region R2 defines $CD34^+CD38^-$ cells. Region R3 defines $CD34^+CD38^+$ cells. Region R4 defines $CD34^+CD38^{dim}$ cells. (B) Histogram showing 7-AAD fluorescence of bone marrow MNC from R1 (Fig 1A). M1 defines cells in G_0/G_1 phase. M2 defines cells in S phase. M3 defines cells in G_2/M phase.

gated from R1 (defined by forward and side scatter in Fig 1) were acquired on listmode data files. To increase the number of events for analysis, 10,000 events gated from the $CD34^+$ cell population were also acquired. Regions defining G_0/G_1 phase, S phase, and G_2/M phase were set using total mononuclear cells as an internal control (Fig 2B). The cell cycling status of the three subpopulations of $CD34^+$ cells (ie, $CD38^+$, $CD38^{dim}$, and $CD38^-$ shown in Fig 2A) was then separately analyzed by determining the 7-AAD fluorescence of cells from each immunophenotypic gate (regions R2, R4, and R3, respectively, in Fig 2A). $CD34^+CD38^{dim}$ cells were those $CD34^+$ cells with CD38-PE expression less than the maximum background fluorescence of the isotype control but greater than half the maximal fluorescence of the isotype control. Chicken erythrocyte nuclei (CEN; DNA QC particle kit; BDIS) were used as a positive control for 7-AAD staining.

Long-term stromal cultures and methylcellulose cultures. Bone marrow stroma was produced by culturing fresh mononuclear bone marrow cells in stromal medium (12.5% horse serum, 12.5% fetal calf serum [FCS; Gemini Bioproducts, Calabasas, CA], Iscoves modified Dulbecco's medium [IMDM, GIBCO BRL, Bethesda, MD], 2-mercaptoethanol (Sigma), 10^{-6} mol/L hydrocortisone [Sigma], penicillin/streptomycin, and glutamine) for at least 3 weeks. Macrophages were depleted from the stromal cultures by trypsinizing and replating at least four times before final use in the long-term cultures. One to 2 days before cell sorting, allogeneic bone marrow stroma was irradiated with 20 Gy and plated (7×10^3 cells/well) in 96-well plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) to form pre-established stromal layers for the long-term cultures. Two hundred to 500 cells (with either the $CD34^+CD38^-$ or $CD34^+CD38^+$ immunophenotype) were sorted into individual wells of the 96-well plates in quadruplicates and cultured on the irradiated stroma in long-term bone marrow culture (LTBMC) medium (IMDM, 30% FCS, 1% bovine serum albumin [BSA; Sigma], 2-mercaptoethanol [Sigma], 10^{-6} mol/L hydrocortisone [Sigma], penicillin/streptomycin, glutamine, and the combination of 10 ng/mL interleukin-3 [IL-3], 50 U/mL IL-6, and 50 ng/mL Steel factor [SF]). Every 2 to 3 weeks, nonadherent cells were removed and counted and aliquots were replated in two different concentrations each in duplicate into semisolid media (1.3% methylcellulose in LTBMC medium with IL-3, IL-6, SF, granulocyte-macrophage colony-stimulating factor [GM-CSF; 50 ng/mL], and 2 U/mL erythropoietin). CFU-C were counted after a further 14 days and the mean of quadruplicates was recorded.

Cloning efficiency of $CD34^+CD38^+$ and $CD34^+CD38^-$ cells. To compare the cloning efficiency of $CD34^+CD38^+$ and $CD34^+CD38^-$ cells from cord blood and bone marrow, single cells were deposited in each well of 96-well plates using the Automated Cell Deposition Unit (ACDU) of the FACSVantage. The culture plates were prepared 1 to 2 days before cell sorting with LTBMC medium containing IL-3, IL-6, SF, and pre-established, irradiated BM stromal layers, as described above. Cultures were fed twice weekly by replacing half the supernatant with fresh LTBMC medium. The plates were counted every 7 days, and wells were recorded as positive if greater than 100 cells were present. Cloning efficiency was defined as the number of positive wells ÷ total wells × 100%.

RESULTS

Frequency of $CD34^+CD38^-$ cells in cord blood and bone marrow. The frequency of $CD34^+CD38^-$ cells in cord blood and bone marrow was determined by FACS analysis using the quadrants defined in Fig 1. The quadrant defining cells negative for CD34 and CD38 was set to include at least 99% of cells stained with the isotype control. The percentage of $CD34^+$ cells was lower in cord blood MNC than in bone marrow (mean, 0.36% of cord blood MNC *v* 1.63% of bone marrow MNC), with a large variation in the cord blood samples (range, 0.02% to 1.43%; Table 1). The frequency of $CD34^+CD38^-$ cells, defined by isotype control quadrants, was similar in cord blood and bone marrow (mean, 0.05% from both sources) because of a relatively higher proportion of $CD34^+$ cells with the $CD38^-$ immunophenotype in cord blood.

Isolation of $CD34^+$ subpopulations by FACS. $CD34^+$-$CD38^-$ cells isolated by FACS sorting for further analysis

and culture were defined as those $CD34^+$ cells with PE-CD38 fluorescence less than one half the PE isotype control (region R2 in Fig 1B). Using this stringent definition, a consistent frequency of $CD34^+CD38^-$ cells was found within the total mononuclear cell fraction from cord blood and bone marrow (0.02% ± 0.01% for both cord blood and bone marrow; Table 1). Region R3 was used to define $CD34^+CD38^+$ cells.

HLA-Dr and lineage-specific antigen expression of cord blood $CD34^+CD38^-$ cells. To determine the HLA-Dr expression of cord blood $CD34^+CD38^-$ cells, MNC were stained with FITC-CD34, PE-CD38, and PerCP–HLA-Dr and analyzed by triple-color flow cytometry. Cells falling in the $CD34^+CD38^-$ R2 gate showed a homogeneously low to negative HLA-Dr expression falling in the proximity of the isotype control marker. The $CD34^+CD38^-$ cells with low HLA-Dr expression comprised less than 10% of the total $CD34^+HLA\text{-}Dr^+$ cells (data not shown).

Cell cycle status of $CD34^+CD38^-$ cells in bone marrow and cord blood. 7-AAD, a fluorescent DNA stain, permits determination of the cell cycle status of rare cells defined by dual cell surface antigen expression. To study the relationship of cell cycle status to CD38 antigen expression in $CD34^+$ cells, we analyzed the 7-AAD fluorescence on each of the three $CD34^+$ subpopulations ($CD38^+$, $CD38^{dim}$, and $CD38^-$) shown in Fig 2A (defined in the Materials and Methods).

In both bone marrow and cord blood, the percentage of $CD34^+$ cells in cell cycle (S-G_2/M phase) increased as CD38 antigen expression increased (Fig 3). A lower percentage of the more primitive $CD34^+CD38^-$ cells was cycling than either the $CD34^+CD38^+$ cells or the $CD34^+CD38^{dim}$ cells. Although there was sample-to-sample variation in the percentage of cells cycling, the positive correlation between CD38 antigen expression and cell cycling was maintained in each sample. A relationship between 7-AAD and PE-CD38 fluorescence was not seen in the $CD34^-$ population, showing that 7-AAD fluorescence was not an artefact of spectral overlap with PE.

A small percentage of $CD34^+CD38^-$ cells (5.4% ± 1.8%, mean ± SD) was cycling in all cord blood samples. These results contrasted with those found for the $CD34^+CD38^-$ cells in bone marrow, in which no cycling was seen in four samples and 2% of cells were in cycle in the fifth sample.

The $CD34^+CD38^-$ phenotype enriches for cells with high and prolonged generative capacity in extended long-term culture. Having established that increasing CD38 expression correlates with an increasing percentage of $CD34^+$ cells in cycle, we chose to use a stringent definition of negative CD38 expression to isolate and assay the functional characteristics of the most quiescent population of $CD34^+CD38^-$ cells. We compared the generative capacity (ie, the ability to generate nonadherent cells and CFU-C) of both $CD34^+CD38^-$ (from R2 in Fig 1B) and $CD34^+CD38^+$ cells (from R3) from cord blood and bone marrow.

The sorted populations were cultured on irradiated bone marrow stroma in the presence of IL-3, IL-6, and SF, a combination that we have previously found to allow maximal cloning efficiency and cell proliferation from primitive progenitors. At 2 to 3-week intervals, aliquots of nonadherent cells were removed from the stromal cultures, counted, and plated in methylcellulose medium to measure the progenitor content of the cultures.

In cultures from both bone marrow and cord blood, the total expansion of nonadherent cells was significantly greater when initiated with $CD34^+CD38^-$ cells than with $CD34^+CD38^+$ cells (Fig 4). The time course of cell expansion was strikingly different between the two subpopulations. Maximal expansion from $CD34^+CD38^+$ cells occurred around day 30. In contrast, cell expansion from $CD34^+CD38^-$ cells peaked later, between days 60 and 80 of culture.

Paralleling the differences in the production of nonadherent cells, there was a marked difference in the level and persistence of CFU-C production by the $CD34^+CD38^-$ cells and $CD34^+CD38^+$ cells. Figure 5 shows representative experiments from a total of five cord blood and seven bone marrow samples. The magnitude of CFU-C expansion varied between experiments, but a pattern of prolonged CFU-C production exclusively from $CD34^+CD38^-$ cells was consistently seen. By extending the period of long-term culture,

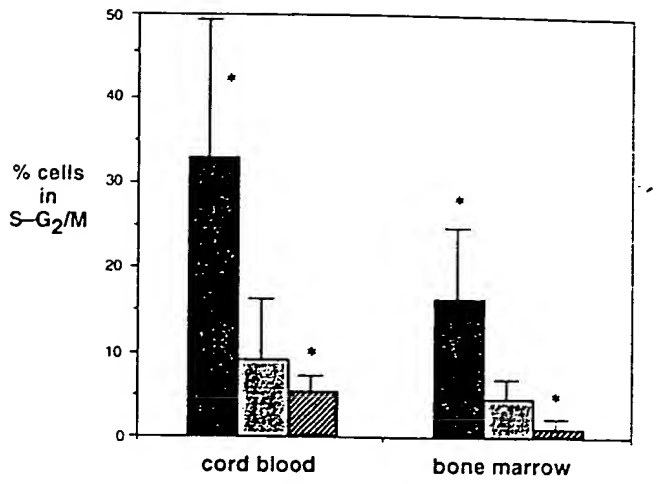

Fig 3. Cell cycle analysis of fresh cord blood and bone marrow $CD34^+$ subpopulations. Using the DNA stain 7-AAD, cell cycle status was determined for each of the (▨) $CD34^+CD38^-$, (□) $CD34^+CD38^{dim}$, and (■) $CD34^+CD38^+$ subpopulations defined by regions R2, R4, and R3 respectively in Fig 2A. The vertical axis shows the percentage of each subpopulation in S-G_2/M (defined by M2-M3 in Fig 2A). The mean ± SD for each subpopulation is shown (n = 5 bone marrow samples, n = 5 cord blood samples). The percentage of $CD34^+CD38^-$ cells in S-G_2/M is significantly higher than the percentage of $CD34^+CD38^-$ cells in S-G_2/M in both cord blood and bone marrow by paired *t*-test (*P = .02).

Table 1. Frequency of $CD34^+$ and $CD34^+CD38^-$ Cells in Cord Blood and Bone Marrow MNC

	% of Total MNC (mean ± SD)	
	$CD34^+$	$CD34^+CD38^-$ (region R2)
Bone marrow (n = 15)	1.63 ± 0.96	0.02 ± 0.01
Cord blood (n = 30)	0.36 ± 0.33	0.02 ± 0.01

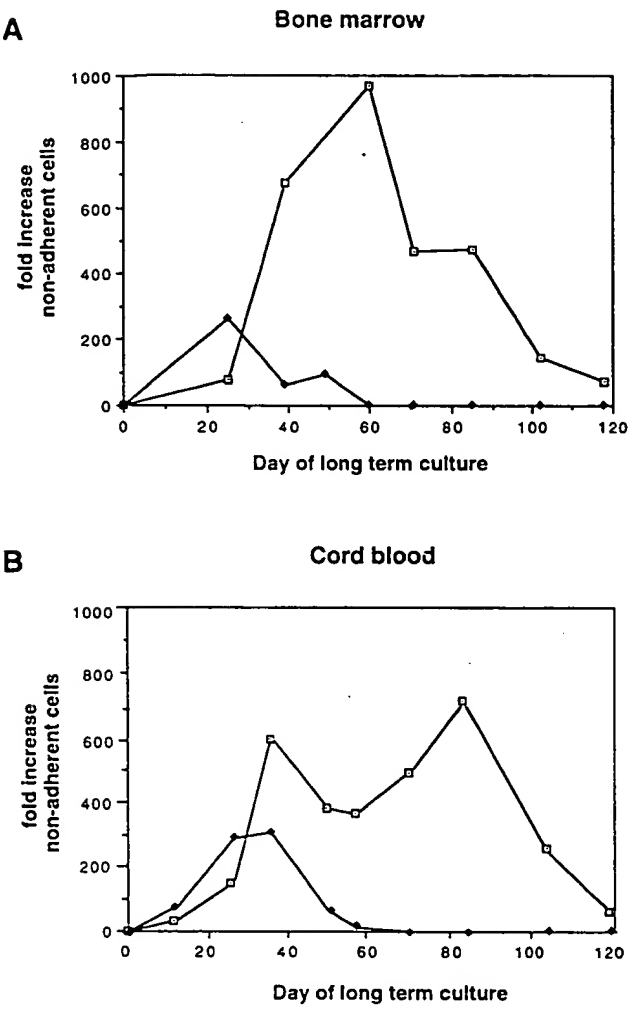

Fig 4. Nonadherent cell expansion from (◆) CD34⁺CD38⁺ and (□) CD34⁺CD38⁻ cells. CD34⁺CD38⁺ and CD34⁺CD38⁻ cells from bone marrow (A) and cord blood (B) were plated in quadruplicate on day 0 by FACS sorting onto pre-established irradiated allogeneic stroma at 500 cells/well in a 96-well plate. Cells were cultured on stroma in LTBMC medium with IL-3, IL-6, and SF (see Materials and Methods). Every 2 to 3 weeks, nonadherent cells were removed from each culture and counted. The mean fold increase over cell number plated on day 0 is shown at each time point of long-term culture.

we were able to compare CFU-C production from each subpopulation during the standard 35- to 56-day period of the LTCIC assay with production during extended long-term culture (E-LTC), ie, between days 60 and 100. We hypothesized that hematopoiesis during E-LTC may be initiated from a more primitive subpopulation of progenitor cells (E-LTCIC) than standard LTCIC.

CFU-C production (both the percentage and the total number of CFU-C) from CD34⁺CD38⁻ cells from both bone marrow and cord blood was higher than that from CD34⁺CD38⁺ cells after 35 days of culture, indicating enrichment of LTCIC in the CD34⁺CD38⁻ population.

The most striking difference in CFU-C production between the CD34⁺CD38⁺ and CD34⁺CD38⁻ cells from both bone marrow and cord blood was seen after 40 days of culture. CFU-C were produced beyond day 40 only from cultures initiated with CD34⁺CD38⁻ cells. CFU-C numbers from each CD34⁺CD38⁻ culture showed a biphasic pattern, with an early peak around day 40 to 50 and a later peak occurring between days 40 and 80. CFU-C production from CD34⁺CD38⁻ cells persisted for 80 to 120 days of culture. The pattern of prolonged CFU-C production from E-LTC distinguished CD34⁺CD38⁻ cells from CD34⁺CD38⁺ cells more clearly than did CFU-C production during the standard LTCIC assay period of 35 to 56 days.

Late appearance of clones from single cord blood CD34⁺CD38⁻ cells in extended long-term culture. To study whether the prolonged CFU-C production from bulk cultures of CD34⁺CD38⁻ cells was due to persistent proliferation from highly clonogenic progenitors or to the sequential recruitment of different subpopulations, the timing of clonal proliferation from individual cord blood CD34⁺CD38⁻ cells was determined. Single cord blood CD34⁺CD38⁻ or CD34⁺CD38⁺ cells were placed by FACS into individual wells of 96-well plates prepared with irradiated bone marrow stroma and IL-3, IL-6, and SF. Each well was recorded as positive if at least 100 cells were visible at any time point. Every 1 to 2 weeks, the appearance of new positive wells was noted to determine the onset of clonal proliferation from each CD34⁺CD38⁻ and CD34⁺CD38⁺ cell. The number of wells in which proliferation first appeared at each time point is shown as a percentage of total wells plated (Fig 6).

All of the clones arising from CD34⁺CD38⁺ cells and most of the clones arising from CD34⁺CD38⁻ cells appeared in the first 21 days of culture. However, 2.9% ± 3.1% (mean ± SD) of all CD34⁺CD38⁻ cells plated on day 0 began to proliferate after 60 days of culture, and 1.9% ± 2.6% of all cells plated initiated proliferation after 80 days (n = 1,344 wells/experiment; 2 experiments). Late-proliferating clones did not arise from the CD34⁺CD38⁻ cells in cord blood.

To assess the secondary replating potential of clones present during the standard LTCIC period (35 to 56 days) and to compare them with those present during the E-LTCIC period, we removed cells from positive wells (ie, those that contained viable hematopoietic cells) at days 45 and 88 and replated them in methylcellulose medium. Thirty-six percent of the wells initiated with CD34⁺CD38⁻ cells were positive at day 45, and 13.2% of the positive wells gave rise to CFU-C. Thus, 4.7% (0.36% × 13.2%) of all CD34⁺CD38⁻ cells plated generated CFU-C in the standard LTCIC assay. By day 88 (in E-LTC), 24.2% of all wells still contained viable cells. Of the positive wells, 2.1% were able to give rise to CFU-C. Thus, 0.5% (0.24% × 2.1%) of all cord blood CD34⁺CD38⁻ cells were able to generate CFU-C in E-LTCIC assay.

Cloning efficiency and generative capacity of CD34⁺CD38⁻ cells from cord blood is greater than that from bone marrow. Having established that the CD34⁺CD38⁻ immunophenotype defines a rare, quiescent, and functionally primitive population in cord blood as it does in bone marrow, we next determined whether there are functional differences between the two sources of CD34⁺CD38⁻ cells. The cloning efficiency (ie, the number of cells able to proliferate in culture) of CD34⁺CD38⁻ cells from cord blood was compared with that from bone marrow. Single CD34⁺CD38⁻ cells from bone marrow and cord blood were sorted and cultured in

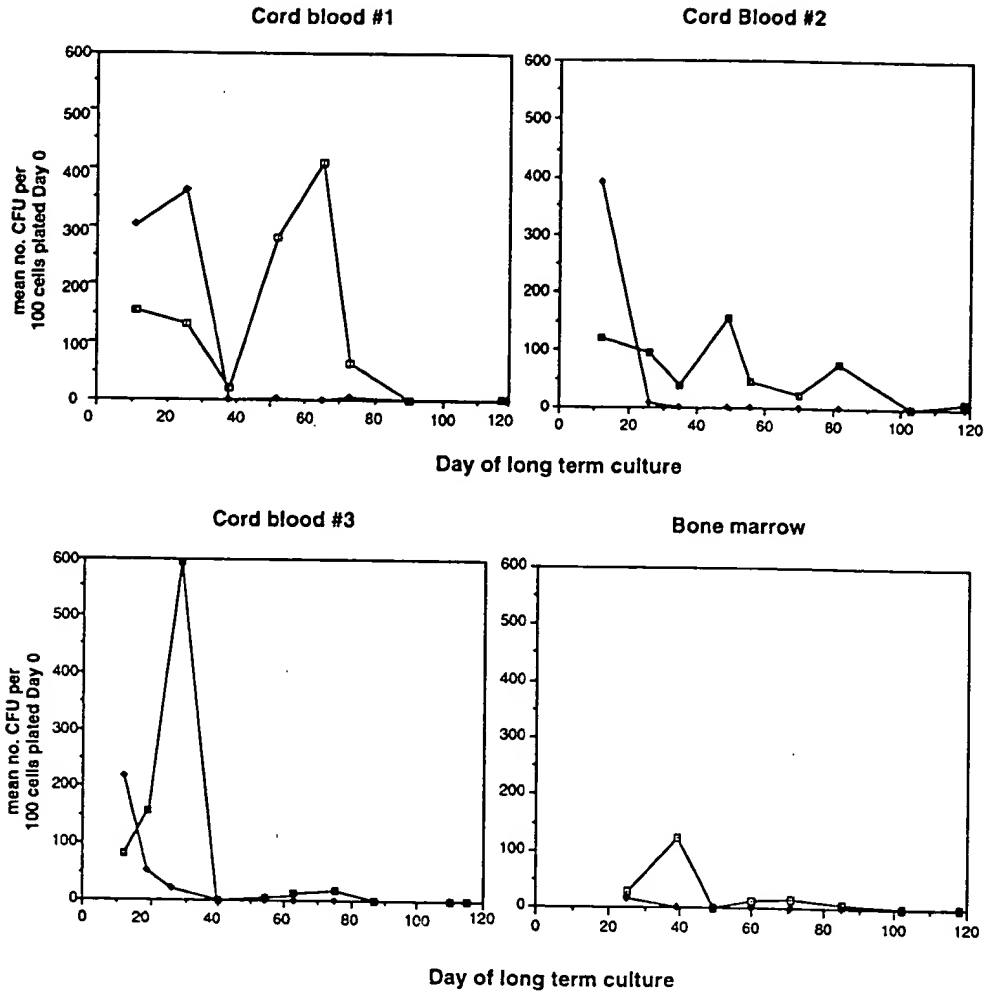

Fig 5. CFU-C expansion from (♦) $CD34^+CD38^+$ and (□) $CD34^+CD38^-$ cells in extended long-term culture. $CD34^+CD38^+$ and $CD34^+CD38^-$ cells were plated in quadruplicate on day 0 by FACS sorting onto pre-established irradiated allogeneic stroma at 500 cells/well in a 96-well plate. Cells were cultured on stroma in LTBMC medium with IL-3, IL-6, and SF. To measure CFU-C production from the long-term cultures, every 2 to 3 weeks nonadherent cells were removed and counted, aliquots were replated in quadruplicates in methylcellulose medium, and CFU-C were counted after a further 14 days of culture. The vertical axes represent CFU-C expansion during long-term culture (calculated as mean percentage of CFU-C per nonadherent cells plated in methylcellulose × mean fold expansion of nonadherent cells [Fig 4] at each time point). Three independent experiments with cord blood and one representative experiment with bone marrow are shown.

IL-3, IL-6, and SF either with or without irradiated stroma. $CD34^+CD38^-$ cells were also sorted as single cells to compare cloning efficiency of the committed progenitors with their primitive $CD38^-$ counterparts. Cloning efficiency was defined as the percentage of wells with at least 100 cells present at day 28.

The cloning efficiency of $CD34^+CD38^-$ cells from cord blood was significantly higher than that of $CD34^+CD38^-$ cells from bone marrow (on stroma, 36.8% ± 2.9% *v* 5.9% ± 0.3%, respectively, $P < .0001$; without stroma, 41.8% ± 9.1% *v* 0.9% ± 0.1%, $P < .0001$; Fig 7). Cloning efficiency of cord blood $CD34^+CD38^-$ cells was not significantly different than that for cord blood $CD34^+CD38^+$ cells. However, in bone marrow, there was a significantly lower cloning efficiency from $CD34^+CD38^-$ cells than from $CD34^+CD38^+$ cells ($P < .0001$).

The effect of stroma on cloning efficiency of $CD34^+CD38^-$ cells differed between cord blood and bone marrow. Cocultivation of cells with bone marrow stroma increased cloning efficiency of bone marrow $CD34^+CD38^-$ cells sixfold ($P = .001$), but did not significantly enhance the plating efficiency of cord blood $CD34^+CD38^-$ cells. The cloning efficiency of $CD34^+CD38^+$ cells from either cord blood or bone marrow was not significantly affected by the presence of stroma.

The onset of proliferation of $CD34^+CD38^-$ cells from cord blood was earlier than from bone marrow $CD34^+CD38^-$ cells. Single $CD34^+CD38^-$ cells from cord blood formed clones of at least 100 cells per well as early as 7 days in culture. In contrast, bone marrow $CD34^+CD38^-$ did not form clones until approximately 21 days (data not shown).

Individual cord blood $CD34^+CD38^-$ cells had a greater generative capacity than those in bone marrow (Table 2). Clones produced from single cord blood $CD34^+CD38^-$ cells contained sevenfold more cells than those from bone marrow. $CD34^+CD38^-$ cells from both bone marrow and cord blood produced respectively 10-fold and threefold more cells than did $CD34^+CD38^+$ cells from each source, again showing the higher generative capacity of the more primitive $CD34^+CD38^-$ cells.

DISCUSSION

These studies show that the $CD34^+CD38^-$ immunophenotype defines a rare and highly primitive population of cells in cord blood. $CD34^+CD38^-$ cells are distinct from the more numerous $CD34^+CD38^+$ progenitors on the basis of their cell cycling status and their capacity to generate progeny over an extended period of long-term culture.

The $CD34^+CD38^-$ immunophenotype is consistent in definining a highly primitive subpopulation of $CD34^+$ cells in

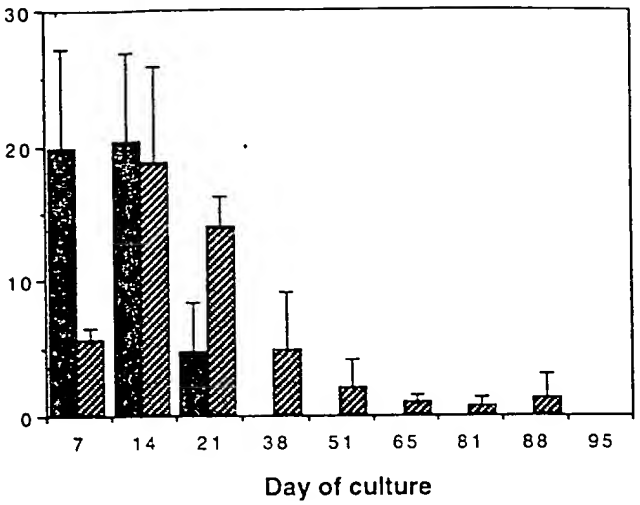

Fig 6. Timing of appearance of new clones from single cord blood (▨) CD34⁺CD38⁻ and (■) CD34⁺CD38⁺ cells. Pre-established irradiated allogeneic stroma was plated in each well of a 96-well plate. Individual CD34⁺CD38⁻ and CD34⁺CD38⁺ cord blood cells were plated by FACS as single cells per well onto stroma using the ACDU of the FACSVantage. For each experiment, a total of 1,344 wells were plated with CD34⁺CD38⁻ cells and 192 wells were plated with CD34⁺CD38⁺ cells. Cells were cultured in LTBMC medium in IL-3, IL-6, and SF (see Materials and methods) for 120 days with twice weekly changes of half the medium. Approximately once per week, all plates were scored for positive wells (100 cells/well plated) and wells that had become positive since the previous scoring were recorded. The vertical axis represents the percentage of all wells plated that became positive at each time point. Mean values ± SD are shown for two independent experiments.

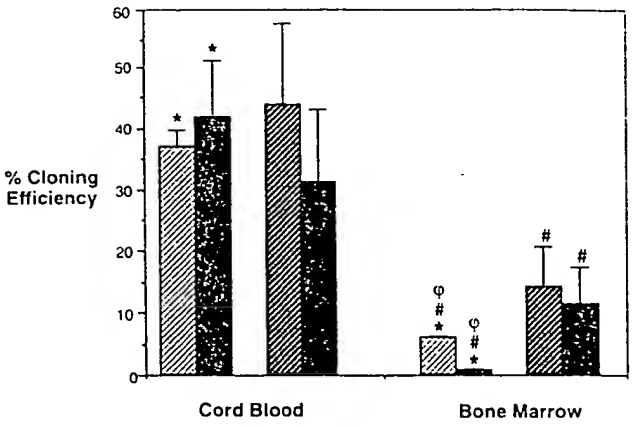

Fig 7. Comparison of cloning efficiency of CD34⁺CD38⁻ and CD34⁺CD38⁺ cells from cord blood and bone marrow. Wells of 96-well plates were prepared with LTBMC medium and IL-3, IL-6, and SF, either with or without irradiated stroma. Individual CD34⁺CD38⁻ and CD34⁺CD38⁺ from cord blood or bone marrow were plated by FACS as single cells per well as described in Fig 6. Cloning efficiency was defined as the percentage of wells with at least 100 cells present at day 28. For each experiment, a mean of 992 wells were plated with CD34⁺CD38⁻ cells and 188 wells were plated with CD34⁺CD38⁺ cells from cord blood and from bone marrow. The results shown are the mean ± SD from three experiments with bone marrow and two with cord blood. *$P < .0001$, significant difference of plating efficiency between CD34⁺CD38⁻ cells from bone marrow and from cord blood (grown either with or without stroma) by paired t-test. †$P < .0001$, significant difference between CD34⁺CD38⁻ cells and CD34⁺CD38⁺ cells in bone marrow ± stroma. ‡$P < .001$, significant difference between bone marrow CD34⁺CD38⁻ cells grown on stroma compared with those grown without stroma. (▨) CD34⁺CD38⁻ with stroma; (■) CD34⁺CD38⁻ without stroma; (▨) CD34⁺CD38⁺ with stroma; (■) CD34⁺CD38⁺ without stroma;

all sources of HSC studied to date, ie, fetal liver, fetal bone marrow, adult bone marrow, and cord blood.[8-12] In contrast, HLA-Dr expression on highly primitive progenitors varies with each hematopoietic source.[6-8,11] Our studies show that CD34⁺CD38⁻ cells in cord blood have homogeneous low HLA-Dr expression.

In these studies, we have defined an immunophenotypically homogeneous population of cells. However, at least two functionally distinct subpopulations exist within the CD34⁺CD38⁻ cells of bone marrow and cord blood, ie, those able to produce CFU-C within the standard LTCIC assay period (5 to 8 weeks) and a more rare subpopulation that begins to proliferate in culture after 8 weeks (the period of extended long-term culture). Although the CD34⁺CD38⁻ population is enriched for CFU-C that appear during the standard LTCIC period, it is the ability to produce CFU-C beyond 8 weeks that most clearly distinguishes the primitive CD34⁺CD38⁻ bone marrow and cord blood cells from the more mature CD34⁺CD38⁺ cells. Consistent with this prolonged progenitor output is the late appearance of blast cell clones arising only from single cells with the CD34⁺CD38⁻ immunophenotype. These late-appearing clones presumably represent the same highly primitive progenitor cells that contribute to the CFU-C during the extended LTCIC period. The purification of functionally distinct subpopulations of CD34⁺CD38⁻ cells by immunophenotypic or other biologic markers will be an important step in the further study of quiescent HSC.

Several observations support the contention that cells assayed during extended long-term culture may represent a different and possibly more primitive population than those measured over the standard LTCIC period. First, the delayed clonogenicity of E-LTCIC suggests a deeply quiescent cell population that is relatively less responsive to cytokine stimulation than the standard LTCIC. Second, using a retroviral vector to mark CD34⁺CD38⁻ bone marrow cells, we have found that the level of transduction of CFU-C produced after 8 weeks of culture (0% to 5%) is significantly lower than transduction of CFU-C during the first 8 weeks of culture (40% to 60%; manuscript in preparation). The lower transduction efficiency of E-LTCIC is consistent with a target population more quiescent than standard LTCIC. A transduction efficiency of less than 5% in E-LTCIC is also compara-

Table 2. Generative Capacity of Individual CD34⁺CD38⁻ and CD34⁺CD38⁻ Cells From Cord Blood and Bone Marrow

	Cell No. From Each Clone (×10⁴)	
Single Cells Plated	Cord Blood	Bone Marrow
CD34⁺CD38⁻	75 ± 33	3 ± 2
CD34⁺CD38⁻	208 ± 302	28 ± 36

Values shown are mean ± SD for bone marrow clones (n = 15) and cord blood clones (n = 59).

ble to that seen for HSC in vivo in large animal studies and clinical human gene therapy trials.[17-20] Finally, a hierarchical system of pluripotent hematopoietic progenitor cells has been described in murine studies with a functional distinction shown between day 12 CFU-spleen (CFU-S) cells and the more quiescent and primitive long-term repopulating cells.[21-23]

Although the $CD34^+CD38^-$ immunophenotype defines a functionally primitive subpopulation in cord blood and bone marrow, important differences exist between the $CD34^+CD38^-$ cells from each source. Consistent with other reports, we found that both committed and primitive progenitors from cord blood proliferate more rapidly in response to cytokine stimulation in vitro than their counterparts in bone marrow.[2,7] The more rapid onset of proliferation in vitro is partly due to the larger percentage of cord blood HSC cycling in vivo but is also because of a greater capacity of quiescent cord blood cells to respond to stimulation. Not only do a significantly higher percentage of cord blood $CD34^+CD38^-$ cells proliferate in vitro, but each cord blood cell (whether $CD34^+CD38^+$ or $CD34^+CD38^-$) can generate almost a log more progeny than its counterpart in bone marrow.

These biologic differences between highly primitive cells in cord blood and bone marrow may have important implications for human gene therapy. The results of human clinical trials using retroviral vector-mediated transduction of bone marrow have been disappointing, with low or absent transduction of long-lived progenitors. The quiescent nature of hematopoietic stem cells in bone marrow is likely to be the major obstacle to efficient retroviral-mediated gene transfer.[24,25] Successful transduction of long-lived progenitors has been achieved in one clinical gene therapy trial using cord blood and in another using bone marrow from patients recovering from chemotherapy.[26,27] In each case, it is likely the increased efficiency of transduction resulted from a higher percentage of cycling HSC. To induce proliferation of HSC from normal bone marrow with the current array of cytokines available, prolonged in vitro stimulation is likely to be necessary, leading inevitably to cell differentiation and loss of stem cell function. The highly proliferative HSC of cord blood may thus provide a more promising target for HSC gene therapy.

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